

## Two new functions of inositol in the eye lens: Antioxidation and antiglycation and possible mechanisms

S Ramakrishnan\*, K N Sulochana and R Punitham

Biochemistry Research Department, Vision Research Foundation, Sankara Nethralaya, 18, College Road,  
Chennai 600 006, India

Received 15 June 1998; revised 26 November 1998

The extent of glycation of human eye lens proteins with glucose in presence of added inositol was examined *in vitro* using [ $^{14}\text{C}$ ] glucose. Lens homogenate was reacted with varying concentrations of glucose and glucose + inositol. At the end of the reaction, the proteins were precipitated with TCA, centrifuged, dissolved in NaOH and the radioactivity was measured. Inositol decreased the glycation by 57-67 %. Pure inositol and glucose suitably labelled with  $^3\text{H}$  or  $^{14}\text{C}$  when reacted and followed by paper chromatography and HPLC showed that glucosyl inositol was present along with unreacted free glucose. Preliminary studies made of the UV spectra of pure inositol (i) when reacted with  $\text{H}_2\text{O}_2$  showed that inositol removed  $\text{H}_2\text{O}_2$  from the reaction mixture (ii) when reacted with arachidonic acid showed that they formed a conjugate. The observations indicate that the antioxidant property of inositol could be the result of its quenching action on reactive oxygen intermediates and conjugate-formation with compounds like arachidonic acid and the antiglycating property due to scavenging of glucose. The antioxidant and the antiglycating properties of inositol may be beneficial in delaying or averting cataract.

The human eye lens contains about 5.9 mM inositol as against 0.1 mM in the aqueous humor<sup>1</sup>, while free inositol is evenly distributed between the cortex and the nucleus, epithelium and capsule have 60 % higher inositol content than the rest of the lens<sup>2</sup>. Inositol levels are different in the lenses of different species<sup>3</sup>, 5.2±0.05  $\mu\text{mol/g}$  wet weight of the lens in humans, 2.3±0.02 in rats and 3.0±0.03 in goats. Inositol molecule has 6 hydroxyl groups of which the one in carbon atom 2 is in the axial position and the rest in equatorial positions shown in the conformation of the substance<sup>4</sup>. Normally, axial hydroxyl group is a reactive group as compared to others. The known functions of inositol in biological systems are: its role as phosphoinositide in biomembranes to maintain their integrity<sup>5</sup>, activation of enzymes like  $\text{Na}^+$ ,  $\text{K}^+$  ATPase<sup>6</sup> and protein kinase<sup>7</sup>, contribution to osmotic pressure<sup>8</sup> as a nonionic osmolyte, second messenger as myoinositol 1, 4, 5 triphosphate<sup>9</sup>, and oxidation to yield xylulose<sup>10</sup>.

Two biochemical aspects in the etiology of cataractogenesis namely, the oxidant challenge by superoxide anion, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl free-radical<sup>1</sup> which oxidise the membrane

lipids and proteins and the non enzymic glycation of lens proteins<sup>12</sup> by glucose especially in conditions of high blood glucose have been known. The antioxidant function of inositol in *in vitro* peroxidation of the lipids of lens and erythrocytes by hydrogen peroxide has been reported by Raj *et al.*<sup>3,13</sup>. In this paper, the possible mechanism for the antioxidant function is being presented.

Formation and progression of cataract may be due to various causes. In conditions of high blood sugar, it could be due to non enzymic glycation of lens proteins by glucose<sup>12</sup>. Being non enzymic, there is no control for this reaction. Glucose continuously reacts with lens proteins and forms Schiff's base which quickly rearranges to the more stable Amadori product. The Amadori products have carbonyl groups and may react further to form intermolecular cross-links or advanced glycosylation end-products<sup>14</sup>. Such cross-links may account for crystallin-aggregation associated with increased light scattering and lens opacity. The glycosylation may also induce conformational changes to the proteins, and thus increase susceptibility to disulphide bond formation, which could contribute to the protein aggregation<sup>15</sup> and cataract.

Inositol being an alcohol can react with glucose to form a hemiacetal<sup>16</sup>. Once glucose is removed as hemiacetal, lens proteins may be saved from

\* Author for correspondence

Fax: 044-8254180

E.mail: MDSAAA35@giasmd01.vsnl.net.in

continued glycation. Studies were also made to find out whether inositol would form an adduct with glucose and lower the extent of glycation of lens proteins *in vitro* by scavenging glucose.

## Materials and Methods

### Chemicals

Thiobarbituric acid and myoinositol were obtained from Sigma Chemicals, USA.  $H_2O_2$  and glucose were of analytical grade. [ $U^{14}C$ ] glucose was obtained from the Bhabha Atomic Research Centre, Mumbai and [ $^3H$ ] inositol from Amersham.

### Lenses

Lenses for the work were obtained from the eyes received from healthy donors shortly after their death.

### UV spectra

To find out the mechanism of antioxidation, UV spectra were recorded in solutions of two different concentrations of inositol viz 0.1 M and 1 M at two different pH to find out  $H^+$  binding, if any, due to ionization of axial hydroxyl (OH) group of inositol.

Reaction of inositol, if any with hydrogen peroxide, an oxidant, was studied by treating a solution of 1 mg of inositol with 300, 150, 75 and 30  $\mu g$   $H_2O_2$  in a total volume of 1 ml and using Beckman DU 640 Spectrophotometer, with wavelength scan made in the range of 200-400 nm in the UV region.

Likewise, reaction of inositol (1 mg) with a polyenoic acid like arachidonic acid (10 and 5  $\mu g$ ) in a total volume of 1 ml was studied taking UV spectra.

### Studies with oxidised glutathione

Effect of added inositol in the conversion of oxidised glutathione (G-S-S-G) to reduced glutathione (GSH) was investigated in *in vitro* incubation systems. Inositol (2, 6, 8 and 10 mg) in phosphate buffer (pH 6.9) was treated with 2 mg of oxidised glutathione each with and without 2 mg NADPH. GSSG and GSH were separated by paper chromatography using *n*-butanol, acetic acid water (4:1:1 v/v). The spots stained with ninhydrin were cut and eluted with aqueous alcohol and absorbance was taken in a Beckman Spectrophotometer DU 640 at 560 nm. The ratio of GSH/GSSG was calculated<sup>17</sup>. The experiment was repeated with added lens homogenate (1 ml containing 100 mg  $dl^{-1}$ ) with and without 2 mg of added NADPH.

### Glycation of lens proteins

For studies on glycation, interaction of inositol (5 and 10 mM) with glucose (2 and 5 mM) labelled with [ $U^{14}C$ ] glucose (2  $\mu Ci$ ) in phosphate buffer pH 6.9 at 37°C and kept for 120 hr with periodical shaking followed by addition of sodium azide as preservative was investigated. Separation of unreacted [ $U^{14}C$ ] glucose from the adduct, if any, was made in paper, using pyridine, isoamyl alcohol and water (10:10:7 v/v). Paper bits of different locations were immersed in liquid scintillant, the radioactivity measured in Liquid Scintillation System (Beckman) and expressed as disintegrations per minute (dpm)<sup>18</sup>.

The products of interaction of inositol with glucose were also tested by reverse phase HPLC, [LKB Pharmacia]. LKB superpak cartridge 4×250 nm, Spherisorb ODS 2.5  $\mu m$  Octadecyl Silane column, milli Q water as a mobile phase with flow rate of 1 ml/min and detection at 188 nm UV 0.02 AUFS were employed<sup>19</sup>. A total volume of 250  $\mu l$  sample containing inositol and glucose was injected and eluted with milli Q water as a mobile phase at the flow rate of 1 ml/min and the radioactivity of the fractions counted in LSS 640 Beckman using scintillant fluid.

The above experiments were done using [ $^3H$ ] inositol (5 mM, 2  $\mu Ci$ ) and glucose.

Extent of *in vitro* glycation of lens proteins for different levels of glucose with and without inositol was investigated as follows: lens homogenate (100 mg  $ml^{-1}$ ) in phosphate buffer, pH 6.9 was treated with glucose, (2 and 5 mM) and [ $U^{14}C$ ] glucose (2  $\mu Ci$ ) incubated at 37°C for 120 hr. Sodium azide was used as preservative. Proteins were precipitated with trichloroacetic acid (TCA), separated by centrifugation, washed free of radioactive contaminants and dissolved in NaOH. Aliquots were taken in liquid scintillant. Extent of radioactivity (dpm) was measured in Liquid Scintillation System (Beckman). The experiment shows the extent of glycation of lens proteins on adding glucose and was repeated with added inositol (5 and 10 mM) to study its possible effect.

## Results

There was no evidence of ionization of -OH group of inositol shown by UV spectral studies on  $H^+$  binding. (Personal communication, Ghosh S K, Saha Institute of Nuclear Physics, Calcutta, Nov. 22, 1995). There was also no significant conversion of GSSG to GSH.

Inositol had a peak at 225 nm while H<sub>2</sub>O<sub>2</sub> had its peak at 237 nm. On mixing the two, the peaks due to both inositol and H<sub>2</sub>O<sub>2</sub> vanished. Adding different concentrations of H<sub>2</sub>O<sub>2</sub> to a fixed concentration of inositol gave the same results suggesting that H<sub>2</sub>O<sub>2</sub> was removed from the system. That the removal of H<sub>2</sub>O<sub>2</sub> gives such a UV curve was confirmed by taking a solution of 300 µg H<sub>2</sub>O<sub>2</sub>, boiling and following the UV spectrum. Very interestingly the boiled sample gave exactly a similar curve as the one given by H<sub>2</sub>O<sub>2</sub> treated with inositol. It is well known that H<sub>2</sub>O<sub>2</sub> is removed by boiling. Again a similar curve was obtained after treating H<sub>2</sub>O<sub>2</sub> with catalase which also removes H<sub>2</sub>O<sub>2</sub>. The above findings indicate that inositol can scavenge H<sub>2</sub>O<sub>2</sub> and exhibit its antioxidant effect.

It was also found that when inositol and arachidonic acid were made to react, the absorbance of inositol increased from 0.0514 to 0.4198 while that of arachidonic acid decreased from 0.3537 to 0.2344. This was also observed for a different concentration of inositol. This suggests an interaction of inositol and arachidonic acid and probable formation of a conjugate. Such a shielding effect of inositol on arachidonic acid might protect the latter from oxidants. The reaction is analogous to conversion of retinol to its ester retinyl palmitate to prevent its loss<sup>17</sup>.

Thus the mechanism of antioxidant function of inositol may be due to its scavenging of H<sub>2</sub>O<sub>2</sub> and probable formation of a conjugate with polyenoic acids and protecting them from oxidants.

#### Adduct formation of inositol with glucose

Paper chromatography and staining the sugars with aniline hydrogen oxalate showed 2 spots with different R<sub>f</sub> values, the higher one (0.35) for free glucose and the lower (0.13) for glycosylated inositol. Using [<sup>14</sup>C] glucose, separating the mixture by paper chromatography, it was found that there were 2 peaks of radioactivity one major corresponding to the unreacted [<sup>14</sup>C] glucose and the other minor due to [<sup>14</sup>C] glucosyl inositol (Table 1).

On the other hand, with [<sup>3</sup>H] inositol and glucose, there was only one peak of radioactivity on chromatography. This is because the R<sub>f</sub> of [<sup>3</sup>H] inositol and glucosyl [<sup>3</sup>H] inositol are overlapping. This is substantiated by the dpm (Table 2); without glucose. It is 5300 for [<sup>3</sup>H] inositol only. With glucose (non radioactive) it is decreased to 3450 because of dilution of the spot with a non radioactive component viz. glucose.

Table 1—Glycation of inositol: Reaction of [<sup>14</sup>C] glucose with inositol

|  | [Values (dpm) mean of 6± SE]                     |                                      |            |
|--|--|--------------------------------------|------------|
|  | Glucose (mM)+ [ <sup>14</sup> C] glucose (2 µCi) | Inositol                             |            |
|  |  | 5 mM                                 | 10 mM      |
|  |  | Distribution of radioactivity in dpm |            |
| Zone with no free or bound glucose                         | 2  | 22±6                                 | 23±5       |
|  | 5  | 20±7                                 | 24±4       |
| Zone corresponding to [ <sup>14</sup> C] glucosyl inositol | 2  | 8314±974                             | 6525±847   |
|  | 5  | 6218±658                             | 7100±878   |
| Zone corresponding to Free [ <sup>14</sup> C] glucose      | 2  | 20746±4740                           | 23064±6360 |
|  | 5  | 21352±5214                           | 19768±4326 |

Table 2—Radioactivity of inositol labelled with [<sup>3</sup>H] inositol (5 mM) with and without glucose [Values (dpm) are mean of 6 ± SE]

| Radioactivity (dpm) without glucose | Radioactivity (dpm) with glucose (5 mM) |
|-------------------------------------|---|
| 5300±124                            | 3450*±109                               |

\* *p*<0.001

HPLC studies confirmed the above findings i.e. 2 fractions of radioactivity for inositol and [<sup>14</sup>C] glucose and one fraction for glucose and [<sup>3</sup>H] inositol.

#### The effect of *in vitro* addition of glucose to lens proteins with and without inositol

Incubation of lens homogenate with glucose *in vitro* increased the extent of glycation of lens proteins (Table 3). When inositol was also added to the homogenate with glucose, the extent of glycation decreased by 57 to 67 % (Table 3). This shows that inositol in the medium spared the lens proteins from glycation to a significant extent (*p*< 0.001 to 0.005). Inositol is capable of scavenging glucose by forming an adduct and protecting lens proteins from glycation. Inositol has thus antiglycating effect also.

#### Discussion

H<sub>2</sub>O<sub>2</sub>, an oxidant formed in the course of metabolism, can form hydroxyl free radical (OH<sup>•</sup>) with ferrous iron (Fenton's reaction) and with superoxide anion (Haber-Weiss reaction)<sup>17</sup>. The hydroxyl free radical can attack the phospholipids of the membrane through lipid-peroxidation. Biomembranes of lenticular cells and erythrocytes are quite susceptible to oxidative damage in view of their tender nature. Hence these cells have to build up a strong antioxidant defence through substances like glutathione, vitamin C, and vitamin E. It is now

Table 3—Glycation of lens proteins *in vitro* with varying concentrations of glucose and the effect of added inositol in human lens  
[Results are expressed as mean  $\pm$  SE of 6 different sets of experiments]

| Glucose (mM) | Inositol (mM) | Radioactivity (dpm) | % reduction of [ $^{14}$ C] uptake | <i>t</i> | <i>p</i> < |
|--------------|---------------|---------------------|------------------------------------|----------|------------|
| 2            | —             | 45362 $\pm$ 5972    | —                                  | —        | —          |
| 2            | 5             | 19674 $\pm$ 2017    | 57                                 | 4.08     | 0.002      |
| 2            | 10            | 17462 $\pm$ 941     | 62                                 | 4.52     | 0.001      |
| 5            | —             | 51448 $\pm$ 7730    | —                                  | —        | —          |
| 5            | 5             | 20415 $\pm$ 3625    | 60                                 | 3.63     | 0.005      |
| 5            | 10            | 16783 $\pm$ 1190    | 67                                 | 4.39     | 0.001      |

experimentally shown that inositol also can function as an antioxidant.

Unlike vitamin E and vitamin C, inositol does not ionize and remove electrons from oxygen free-radicals like hydroxyl free radical. It does not also convert GSSG to GSH. The mechanism of antioxidant function of inositol appears to be its reaction with of H<sub>2</sub>O<sub>2</sub> nonenzymically and scavenging the latter. Inositol also forms a conjugate with polyenoic acids like arachidonic acid and may shield them from easy oxidation.

Ruf *et al.*<sup>20</sup> have suggested that myoinositol could function as an antioxidant. They treated rats with myoinositol triphosphate for 7-8 weeks and observed a significant lowering of malondialdehyde in blood. The antioxidant nature of inositol has been reported by Hallman *et al.*<sup>21</sup> and Claxson *et al.*<sup>22</sup>. Beyer-Mears *et al.*<sup>23</sup> reported that dietary myoinositol provided a partial protective effect on lens transparency in diabetic rats, similar to that reported for antioxidants like vitamin E<sup>24</sup> and butylated hydroxy toluene<sup>25</sup>.

Inositol is also found to be an antiglycating substance. Scavenging of glucose by inositol is perhaps achieved by hemiacetal formation. The uncontrolled non-enzymic glycation of lens proteins by excessive amounts of glucose in a diabetic lens could be mitigated by inositol.

Transport of myoinositol across the biomembrane is competitively inhibited by extracellular glucose<sup>26</sup>. Hence a decreased transport of myoinositol across the membrane would also lead to decreased intracellular concentration of inositol. Decrease in inositol would affect the functional efficiency of enzymes Na<sup>+</sup>, K<sup>+</sup> ATPase<sup>6</sup> and protein kinase<sup>7</sup>. Inositol is needed for the activation of these enzymes. The mechanism could be that glycation of enzyme-proteins<sup>27</sup> is decreased by inositol. Diminished efficiency of sodium potassium pump<sup>17</sup> would lead to accumulation of sodium and water in the lens which condition is cataractogenic.

Beneficial effects of inositol in cataract have already been reported by different groups of workers. As indicated earlier, (i) dietary myoinositol provided a partial protective effect on lens transparency in diabetic rats<sup>23</sup>. (ii) Ruf *et al.*<sup>20</sup> fed rats with myoinositol 1,4,5 triphosphate for 7 to 8 weeks and found the incidence of cataract reduced by 28 to 44 %. (iii) A commercial preparation to mitigate cataract was tried by Phakan Laboratories, Chauvin Blanche<sup>12</sup>. It consisted of inositol, ascorbic acid, pyridoxine, arginine and the three amino acids-glutamic acid, glycine and cysteine-the components of glutathione. Phakan trials had a beneficial effect in cortical, nuclear and mixed cataracts. The trials were incomplete. The ingredients used by Phakan were either antioxidants (ascorbic acid and amino acids of glutathione) or antiglycating substances (pyridoxine, arginine and amino acids). It is shown experimentally that inositol is both an antioxidant<sup>3,13</sup> and antiglycating substance<sup>28,29</sup>. Hence it is very likely that it may avert or delay cataract and could be given with glutathione, vitamin C, E and free amino acids like lysine and glycine<sup>30</sup> in suitable proportions. Inositol is non toxic and is present in all living systems.

## References

- 1 Anderson R E (1983) in *Biochemistry of the Eye* pp. 85, Manuals Programme, American Academy of Ophthalmology, California
- 2 Kabasawa I, Lov M F, Merrola L O & Kinoshita J H (1974) *Ophthalmol Res.*
- 3 Raj D G, Ramakrishnan S & Shyamala Devi C S (1994) Poster presentation at the *Sixteenth International Congress of Biochemistry and Molecular Biology*, New Delhi, India, Abstract, P8-56, pp330
- 4 Parthasarathy R & Eisenberg F J (1986) *Biochem J* 235, 313-322
- 5 Mitchel R H (1975) *Biochim Biophys Acta* 415, 81-87
- 6 Greene D A, Lattimer S A & Sima A A I (1988) *Diabetes* 37, 688-693
- 7 Nishizuka Y (1984) *Science* 225, 1365-1370

- 8 Thurston J H, Sherman W R, Hauhart R E & Kloepper R F (1989) *Paediatric Res* 26, 482-485
- 9 Berridge M J (1987) *Annu Rev Biochem* 56, 159-193
- 10 Goode D & Crabbe M J C (1994) *Exp Eye Res* 59 (Suppl.), 1: S 157
- 11 Duncan G (1981) in *Mechanisms of cataract formation in the human lens*,
- 12 Harding J J (1991) *Curr Opin Ophthalmol* 2, 3-15
- 13 Raj D G, Ramakrishnan S & Shyamala Devi C S (1995) *Indian J Biochem Biophys* 32, 109-111
- 14 Frank R N (1991) *Ophthalmology* 98, 586-93
- 15 Beswick H T & Harding J J (1987) *Biochem J* 246, 761-9
- 16 Lehninger A L (1990) in *Principles of Biochemistry*. 2nd ed, pp 282,
- 17 Ramakrishnan S (1992) in *Essentials of Biochemistry and Ocular Biochemistry*. pp. 28-29; 47-48; 52-54; 79-80 Annamalai University Publications Division, Annamalaiagar
- 18 Ramakrishnan S, Sulochana K N, Punitham R & Arunagiri K (1996). *Glycoconjugate J* 13, 519-523
- 19 Sulochana K N, Ramakrishnan S, Vasanthi S B, Madhavan H N, Arunagiri K & Punitham R (1997) *Br J Ophthalmol* 81, 319-323
- 20 Ruf J C, Ciavatti M, Gustafsson T & Renaund S (1992) *Biochem Med Metab Biol (USA)* 48, 46-55
- 21 Hallman M, Pohjavuori M & Bry K (1990) *Lung* 168 (Suppl.), 877-882
- 22 Claxson A, Morris C, Siren M J, Blake D, Halliwell B, Gustafsson T, Lofavist B & Bergelin I (1990). *Agent Action* 20, 68-70
- 23 Beyer-Mears A, Bucci F A J, Delval M & Cruz E (1989) *Pharmacol* 39, 59-65.
- 24 Ross W M, Creighton D, Stewart-Dettaan P J, Sanwal, Hirst M & Trevithick J R (1982) *Canadian J Ophthalmol* 17, 61-66
- 25 Linklater H A, Dzialorzynski T, Mmcleod H L, Sanford S E & Trevithick J R (1986) *Exp Eye Res* 43, 305-313
- 26 Mistry K P, Beyer-Mears A & Diecke F P J (1993) *Diabetes* 42, 1737-44
- 27 Blakytyn R & Harding J J (1992) *Biochem J* 228, 303-307
- 28 Ramakrishnan S, Sulochana K N, Punitham R & Badrinath S S (1995) Poster presentation at *Association for Research in Vision and Ophthalmology (USA)*, *Investigative Ophthalmal and Visual Science*, Abstract 36, 4050-31
- 29 Ramakrishnan S (1995) *Symposium in Alcon Laboratories*, Texas, May 1995
- 30 Ramakrishnan S & Sulochana K N (1993) *Exp Eye Res* 57, 623-628